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Comparison of Indirect Hemagglutination and Indirect Immunofluorescence Tests with Microneutralization Tests for Detection of Type-Specific *Herpesvirus hominis* Antibody

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Indirect hemagglutinating and immunofluorescent antibody responses to *Herpesvirus hominis* types 1 and 2 were compared to neutralizing antibody responses in infected humans from whom *H. hominis* type 1 or 2 was isolated. The indirect immunofluorescent antibody test was shown to be the most sensitive and specific for primary human infections. The sensitivity and specificity of the indirect hemagglutination and the immunofluorescent antibody tests were shown to be equal to that of the microneutralization test among patients who had primary or recurrent *H. hominis* type 2 infections. It is suggested that the indirect hemagglutination test is preferable for assaying large populations for previous infection with *H. hominis* type 2 because it is rapid, easier to perform, and more economical. The intermediate range of titer differences (Δt) between *H. hominis* types 1 and 2 previously reported to be due to infections with both viruses was shown to occur in all three tests among patients with primary infections with either virus.

The epidemiological association of cervical neoplasia with past infection with *Herpesvirus hominis* type 2 (HVH-2) is based upon a comparison of the incidence of humoral antibodies to HVH-2 in the sera of patients and matched controls (4, 15, 17, 18, 19, 21, 23). Therefore, it is important that the test employed be sensitive and specific. Tests in current use, however, including microneutralization (MNT), kinetic neutralization, and plaque reduction, are all plagued by the fact that there is cross-neutralization between HVH-1 and HVH-2 (16). Furthermore, the tissue culture procedures for plaquing and neutralization are time consuming and expensive.

The indirect hemagglutination test (IHAT) (2) and the indirect immunofluorescent antibody test (IFAT) (11) also exhibit cross-reaction between types 1 and 2. By contrast, however, these tests require no tissue culture other than that for the propagation of antigen, and the results are obtained in 1.5 and 3 h, respectively. If these tests were equally sensitive, and specific and easier to perform, their use would facilitate epidemiological studies in which large numbers of patients and controls are to be tested.

This paper compares the sensitivity and the specificity of the MNT, IHAT, and IFAT in patients with symptoms of HVH infection from whom virus isolates were obtained and typed.

MATERIALS AND METHODS

Virus and serum typing by MNT were performed at the Virus Research Unit of Children's Hospital Medical Center. Serum typing by IHAT was performed at the Infectious Disease Branch of the National Institute of Neurological and Communicative Disorders and Stroke, and that by IFAT was performed at the New England Regional Primate Research Center.

Source of viruses and serum samples. Prototype viruses, the MacIntyre VR-3 strain (HVH-1) and the MS strain (HVH-2), were obtained from the American Type Culture Collection, Rockville, Md. and propagated in a line of human foreskin cells (FS-9) initiated at the Center for Disease Control for the MNT. They were obtained from Andre Nahmias and propagated in a line of human skin cells (MA-196, Microbiological Associates) for the IHAT and in a line of owl monkey kidney cells (OMK 689) initiated at the New England Regional Primate Research Center for the IFAT.

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Standard control rabbit antisera employed for typing viruses at Children's Hospital Medical Center were reference sera VR_3 (HVH-1) and MS (HVH-2) from the Center for Disease Control. Fluorescein-conjugated rabbit antiserum to human immunoglobulins was obtained from Clinical Sciences (lot no. 31701).

Virus isolates and serum samples were obtained from 30 patients during acute and convalescent stages of primary and/or recurrent herpetic infections. The virus type, nature of the infection, and location of the lesions, together with the number of virus isolates tested by MNT and the number of sera tested by MNT, IHAT, and IFAT are shown in Table 1.

Virus typing. Isolates from patients were typed by MNT (25) after a single passage in FS-9 cells or primary human embryonic kidney cells by using standard control rabbit antisera to HVH-1 and HVH-2.

Test procedures. MNTs were performed as previously described (25). Briefly, twofold dilutions (beginning at 1:5) of test sera (inactivated at 56°C for 30 min) together with prototype viruses HVH-1 or HVH-2, diluted to approximately 200 50% tissue culture infective doses, were added in 10 replicates into wells of microtiter plates. The plates were incubated for 30 min at 36°C. Equal quantities of a suspension of human foreskin cells in growth medium were added to the wells and incubated for 5 days. Controls consisted of (i) standard HSV-1 and HSV-2 rabbit antisera, (ii) serum diluent with virus and cells, and (iii) test sera with viral diluent and cells. Fifty-percent end point neutralization titers were calculated by the method of Karber (10) and subsequently corrected for a standard virus input of 100 50% tissue culture infective doses (25). Results were expressed as t_c = log 50% end point neutralization titer, corrected to 100 50% tissue culture infective doses of virus input.

IHATs were performed as previously described (7). Optimal dilutions of sensitizing antigens were determined by "box" titration of sheep erythrocytes sensitized with varying dilutions of antigen against varying dilutions of known positive HVH-1 and HVH-2 human antisera whose titers had been determined previously by microneutralization. The working dilution of antigen was that which produced the highest antibody titers for the homologous serum and a fourfold difference between the homologous and heterologous sera.

Equal volumes of tanned sheep erythrocytes, sensitized with HVH-1 or HVH-2, and twofold dilutions of inactivated test sera were incubated in microwells for 1.5 h at room temperature. Controls consisted of serum diluent with sensitized cells and test serum with antigen-free cells.

Test results were interpreted as the highest dilution of serum which gave a 3+ agglutination on a scale of 1 to 4+. Tests were performed in duplicate and simultaneously for HVH-1 and HVH-2.

IFATS were performed as previously described (6). Monolayers of cells from a continuous owl monkey kidney cell line were grown to confluence on sterile glass slides which had been divided into 10 areas by spraying with silicone (Fluroglide, Chemplast, Wayne, N.J.) on a specially constructed brass template. The cells were infected with 200 50% tissue culture infective doses of HVH-1 or HVH-2 and fixed in acetone when discrete focal lesions occurred over 15 to 20% of the monolayer.

Serial twofold dilutions of test sera in phosphate-buffered saline, pH 7.4, were added to the infected cells, incubated for 30 min at 37°C, washed, and then dried with hot air. (Test sera were not treated to inactivate complement [9]. In a separate series of experiments, it was shown that heating the serum

TABLE 1. *Source of viruses and serum samples*

Virus typed by MNT	Nature of infection	No. of patients	Site of lesion (no.)		No. of sera tested by MNT, IHAT, and IFAT	
HVH-1	Primary	6	Oral	(1)	Acute	6
			Meninges	(1)		
			Brain	(1)	Convalescent	7
			Finger	(1)		
			Vulva	(2)		
	Recurrent	6	Lip	(2)		8
			Tongue	(1)		
			Chin	(1)		
			Brain	(1)		
			Vulva	(1)		
HVH-2	Primary	4	Vagina	(1)	Acute	4
			Vulva	(2)		
			Buttocks	(1)	Convalescent	5
	Recurrent	14	Cervix	(1)		20
			Vagina	(1)		
			Vulva	(4)		
			Perineum	(2)		
			Hand	(2)		
			Buttocks	(1)		
			Penis	(1)		
			Cutaneous	(1)		
			Meninges	(1)		

reduced the fluorescent antibody titer significantly in only 1 of 22 sera tested. Addition of 5 U of guinea pig complement to treated serum did not change fluorescent antibody titer in a consistent manner.) Fluorescein-conjugated anti-human globulin, at four times the concentration of the end point, was added, incubated for 30 min, and washed, dried, and mounted under fluorescent antibody mounting fluid (Difco Laboratories, Detroit, Mich.) and examined with a fluorescent microscope. Controls consisted of serum diluent with infected cells and test serum with uninfected cells. Test results were expressed as the highest dilution of serum which gave a 2+ fluorescence on a scale of 1 to 4+. The HVH antigens were shown to be specific because no immunofluorescence was observed with human sera containing antibodies to cytomegalovirus, Epstein Barr virus, or varicella.

Interpretation of tests. Type specificity was determined by two criteria previously established for the interpretation of microneutralization tests. The first criterion was the difference in titers to HVH-1 and HVH-2 (Δt or Δt_c [25]). In all three tests the end points of titration were expressed as $\log_{10}$ of the titers; in the MNT the titers (t_c) were corrected for a standard virus input ($\Delta t = \log_{10} t_1 - \log_{10} t_2$).

Tests in which Δt in IHAT and IFAT or Δt_c in MNT were less than 0.05 were interpreted as type 2 antibody responses; values greater than 0.50 were interpreted as type 1 responses; values between 0.05 and 0.50 were interpreted as intermediate responses (13).

The Δt values could not be determined in sera in which the antibody titer to one virus was less than the lowest dilution tested and that to the other virus was equal to the lowest dilution tested (14).

The second criterion used was the 2/1 index (20). The relationship of the antibody titers to the two viruses was considered as a ratio: ($\log_{10}$ antibody titer to type 2/$\log_{10}$ antibody titer to type 1) $\times$ 100 = 2/1 index.

Sera which failed to show antibody titer at the lowest dilution tested to either virus were considered to be free of antibody, and no index was expressed. In those sera where antibody activity was demonstrated against one but not the other virus, the 2/1 index was calculated by using the lowest dilution tested in the nonreactive mixture.

A 2/1 index equal to or greater than 85 was interpreted as a type 2 response; an index less than 85 was interpreted as a type 1 response.

RESULTS

Primary HVH-1 and HVH-2 infections. Titer differences and 2/1 indexes in convalescent serum samples from patients with primary type 1 and type 2 infections tested by MNT, IHAT, and IFAT are shown in Table 2, and the sensitivity and specificity are compared in Table 3. These patients had no previous episode of herpetic infection by history and absent or low antibody titers in their acute sera (data not shown).

In primary HVH-1 infections, homologous antibodies were present in all sera tested by the MNT and the IFAT and in six of seven sera tested by IHAT (Table 3).

Specificity, as determined by the titer differences and 2/1 indexes, was highest in the IFAT. The IHAT was unable to type sera from patients with primary type 1 infections. Intermediate reactions occurred in all three tests, but there were no heterologous responses in the MNT or the IFAT.

In HVH-2 infections, homologous antibodies were present in all sera in all three tests. Titer differences were type specific only in IFAT. By 2/1 indexes, however, all tests were type specific.

Recurrent HVH-1 and HVH-2 infections. Among patients with HVH-1 infections, the geometric mean titer (GMT) to HVH-1 in each of

TABLE 2. *Comparison of IHAT and IFAT with MNT for serum typing in humans with primary infections*

Virus isolation	Patient no.	Days after first symptoms	MNT		IHAT		IFAT	
			Δt_c	2/1 Index	Δt	2/1 Index	Δt	2/1 Index
HVH-1	38	14	0.05[a]	97[b]	ND[c]	100[b]	0.31[a]	79
	40	14	0.74	57	ND	100[b]	1.50	50
	35	19	0.17[a]	88[b]	0.31[a]	79	0.31[a]	79
	39	37	0.79	73	NA[d]	NA	0.61	66
	37	51	0.41[a]	70	ND	100[b]	0.61	60
		105	0.68	55	0.30[a]	75	1.00	55
	36	67	0.68	69	0[b]	100[b]	0.61	66
HVH-2	25	22	−0.14	108	0	100	−0.31	115
	27	22	0.30[a]	87	−0.30	120	−0.61	140
	28	25	0.09[a]	96	−1.21	234	−0.90	150
		39	−0.24	111	−1.00	192	−0.30	114
	26	120	−0.41	123	0.13[a]	94	0	100

[a] Intermediate response.
[b] Heterologous response.
[c] ND, Not determined ($t_1 = 8$; $t_2 < 8$).
[d] NA, No antibody.

the three tests was more than fourfold higher than the GMT HVH-2 (Table 4).

Among patients with HVH-2 infections, however, GMTs to HVH-2 were only slightly higher than the GMTs to HVH-1 in the MNT and IFAT, but they were twofold higher in the IHAT (Table 5).

In recurrent HVH-1 infections, intermediate responses occurred in sera typed by MNT and IFAT, but not in the IHAT. 2/1 Indexes, however, were type specific in all sera tested by MNT and IHAT (Table 6).

In HVH-2 infections, intermediate reactions occurred in the sera typed by all three tests. Titer differences were specific in 70% of sera tested by the MNT, 75% by the IHAT, and 85% by the IFAT. 2/1 Indexes were 95% specific by the MNT and 90% specific by the IHAT and IFAT.

DISCUSSION

The Δt criteria for serum typing established for the MNT are not comparable for evaluating the IHAT and IFAT. In either of these tests, when sera are titered by twofold dilutions, there is only one opportunity for the titer differences to fall within the intermediate range; that is an HVH-1 titer that is twofold higher than the HVH-2 titer ($\Delta t = 0.30$). Thus, specificity may appear to be higher in the IFAT and IHAT than in the MNT because there are fewer intermediate reactions. More important, however, is the observation that heterologous reactions occurred in both the IHAT and IFAT, whereas there were no heterologous reactions in the MNT.

Based on microneutralization titers in HVH-infected rabbits, Nahmias has proposed that an intermediate range of titer differences between HVH-1 and HVH-2 antibodies represents "dual antibodies" indicative of previous infection with both viruses (13).

In this series of tests and in those of other investigators (1, 20, 25), intermediate reactions

TABLE 3. *Comparison of sensitivity and specificity of antibody assays by MNT, IHAT, and IFAT in convalescent serum samples from patients with primary HVH-1 and HVH-2 infections*

Infection	Test	Sera tested (no.)	Homologous antibodies present (no.)	Differences in titers (Δt)				2/1 Indexes	
				Type 1 response (no.)	Intermediate (no.)	Type 2 response (no.)	Specificity (%)	Homologous response (no.)	Specificity (%)
HVH-1	MNT	7	7	4	3	0	57	5	71
	IHAT	7	6	0	2	4[a]	0	2	33
	IFAT	7	7	5	2	0	71	7	100
HVH-2	MNT	5	5	0	2	3	60	5	100
	IHAT	5	5	0	1	4	80	5	100
	IFAT	5	5	0	0	5	100	5	100

[a] In three of the four sera, the Δt could not be determined ($t_1 = 8$, $t_2 < 8$).

TABLE 4. *Comparison of serum typing in patients with recurrent HVH-1 infections*

Patient no.	MNT		IHAT		IFAT	
	Δt_c	2/1 Index	Δt	2/1 Index	Δt	2/1 Index
1	0.65	74	0.90	65	0.61	66
2	0.59	80	1.20	56	1.81	42
3	0.27[a]	84	0.60	72	0.31[a]	79
	0.39[a]	78	0.60	72	−0.31[b]	125[b]
4	0.91	59	0.60	80	0.61	66
	0.94	59	0.80	73	0.61	60
5	1.09	58	0.60	80	1.50	50
6	0.58	75	0.90	67	0.31*	71
Mean	0.67 ± 0.28[c]	71 ± 11	0.78 ± 0.22	71 ± 8	0.68 ± 0.68	70 ± 25
GMT[d]						
HVH-1	166		324		85	
HVH-2	35		74		18	

[a] Intermediate response.
[b] Heterologous response.
[c] Standard deviation.
[d] GMT expressed as the reciprocal of the titer.

TABLE 5. *Comparison of serum typing in patients with recurrent HVH-2 infections*

Patient no.	MNT		IHAT		IFAT	
	Δt_c	2/1 Index	Δt	2/1 Index	Δt	2/1 Index
7	−0.05	103	0	100	−0.18	115
8	−0.16	111	−0.18	120	−0.30	114
9	−0.21	114	−1.08	190	0	100
	−0.21	113	0	100	−0.61	151
12	0.34[a]	82[b]	0.43[a]	86	0.91[b]	57[b]
14	0.08[a]	97	0.30[a]	86	−0.43	126
	−0.04	102	0.30[a]	83[b]	−0.30	114
15	0.07[a]	97	1.20[b]	64[b]	1.50[b]	55[b]
18	−0.22	113	−0.48	140	−0.48	153
19	−0.04	102	−1.07	171	−0.60	128
20	−0.31	126	−0.60	130	−1.20	179
	−0.58	151	−0.60	132	−0.90	185
21	−0.07	106	−0.60	143	−0.30	128
	0.70[a]	96	−0.90	154	−0.20	109
	0.26[a]	87	−0.90	156	−0.31	126
22	0.02	99	0	100	0.10[a]	94
23	0.14[a]	94	−0.42	125	0	100
24	−0.02	101	0.12[a]	96	0	100
28	−0.03	101	−0.90	143	−0.61	151
	−0.24	110	−0.91	146	−0.30	114
Mean	−0.06 ± 0.20[c]	105 ± 15	−0.32 ± 0.60	123 ± 33	−0.21 ± 0.58	120 ± 33
GMT[d]						
HVH-1	85		85		58	
HVH-2	98		173		85	

[a] Intermediate response.
[b] Heterologous response.
[c] Standard deviation.
[d] GMT expressed as the reciprocal of the titer.

TABLE 6. *Comparison of type specificity of MNT, IHAT, and IFAT in recurrent HVH infections*

Type of infection	Test	Total sera tested	Differences in individual titers				2/1 Index	
			Type 1 response	Intermediate response	Type 2 response	Specificity (%)	Homologous response	Specificity (%)
HVH-1	MNT	8	6	2	0	75	8	100
	IHAT	8	8	0	0	100	8	100
	IFAT	8	5	2	1	63	7	88
HVH-2	MNT	20	0	6	14	70	19	95
	IHAT	20	1	4	15	75	18	90
	IFAT	20	2	1	17	85	18	90

were seen to occur in the MNT in convalescent sera of humans with primary infections. Thus, intermediate reactions are observed when infection with only one virus has occurred. In such sera, the intermediate range of titer differences is a reflection of cross-reacting antibodies, and the sera cannot be typed by titer differences (25).

Thus, by using Δt as a criterion, the MNT is the most accurate for clinical diagnosis if the intermediate range of Δt is interpreted to indicate either dual infection or the presence of cross-reacting antibodies. In the IHAT and IFAT, however, Δt is inappropriate as a criterion for serum typing.

Primary infections. The IFAT was shown to be sensitive and specific, by using the 2/1 index, in typing sera from patients with primary HVH-1 and HVH-2 infections. Fluorescent antibodies appeared earlier than neutralizing or hemagglutinating antibodies, and, in convalescent sera, homologous titers were fourfold higher than heterologous titers in type 1 infections and at least twofold higher in type 2 infections.

Hanna also found the IFAT more sensitive and more specific than the MNT in typing sera from patients with primary herpetic infections (9). Leinikki found antibodies to both viruses appeared within the first week of type 1 or type 2 infection, and homologous titers were at least

fourfold higher in convalescent sera in type 1 infections and equal or twofold higher in type 2 infections (11).

The IHAT was unable to diagnose primary type 1 infections in humans. Titers to HVH-1 were low or absent. These results are similar to those of Back, who reported that HVH-2 antibodies developed more rapidly than HVH-1 antibodies in patients with nongenital infections (1). Fuccillo, however, in an earlier study, found homologous titers fourfold higher than heterologous titers in sera that showed complement fixation seroconversion and which were type 1 by microneutralization (7).

The MNT was not highly specific for serum typing in primary HVH-1 infections by using either the Δt_c or the 2/1 index. Although there were no heterologous responses by Δt_c, there were intermediate responses, particularly in the first weeks after infection.

Detection of type 2 antibodies. Rawls observed that the differences between the HVH-1 and HVH-2 titers in neutralization tests were partly dependent upon the antibody potency and proposed that the relationship of the two titers should be considered as a ratio expressed as the 2/1 index (20). In the MNT, he observed that the 2/1 index was 100% specific in primary type 2 infections and 93 to 95% specific in patients with recurrent type 1 or type 2 infections who had no history of previous heterologous infection (20).

Our series confirms these findings in the MNT and shows that in the IHAT the 2/1 index was 100% specific in primary type 2 infections and recurrent type 1 infections and 90% specific in recurrent type 2 infections. Thus, type specificity by 2/1 index is comparable in the MNT and the IHAT. The disadvantage of the 2/1 index, as defined by Rawls, is that no range of indexes was established to represent the presence of antibodies to both viruses or of cross-reacting antibodies.

It has been shown that approximately 80% of the antibodies to HVH-1 and HVH-2 viruses cross-react with the heterologous virus, whereas 20% are specific for the homologous virus (24). These specific antibodies have been demonstrated by virus neutralization (22), hemagglutination (2, 3), immunofluorescence (8, 12), and cytolysis (24) after absorption of sera with the heterologous virus. The detection of these specific antibodies is necessary to determine whether patients have been infected with one or both viruses. Because cross-absorption is a tedious procedure, it is important to know how well serum typing by the MNT, IHAT, and IFAT correlates with the presence of specific antibodies.

Smith and co-workers, by using cross-absorption in the ^{51}Cr release test, found that sera containing no type 2 specific antibodies had a neutralization 2/1 index of ≤ 80, and sera containing only type 2 specific antibody had an index of ≥ 101 (24). This observation defines a range of 2/1 indexes from 81 to 100 in the MNT which is comparable to the intermediate range of titer differences. In the MNT, approximately 52% of the sera with indexes within this range had no specific antibodies to type 2 demonstrable after cross-absorption with type 1 virus, and 48% did have type 2 specific antibodies.

Back has demonstrated the higher type-specificity of the IHAT in sera with specific type 2 antibodies (2). Eighty percent of these sera had homologous hemagglutinating titers that were fourfold higher than heterologous titers, whereas homologous neutralizing titers were fourfold higher in only 10% of these sera. In sera containing only type 1 specific antibodies, 50% of the 2/1 indexes in both the MNT and the IHAT indicated a type 2 response. Thus, in sera shown to contain specific antibodies, the MNT and the IHAT have approximately the same error in detecting specific type 1 antibodies, but the IHAT may be superior to the MNT in detecting specific type 2 antibodies. No correlation between the IFAT and type-specific antibodies in humans has been made (5).

Conclusions. The Δt is an inappropriate criterion for serum typing by the IHAT and the IFAT. The Δt_c is highly specific in the MNT if the intermediate range is interpreted to mean either dual infection with HVH-1 and HVH-2 or the presence of cross-reacting antibodies.

The IFAT was the most specific in typing primary HVH-1 and HVH-2 infections. The 2/1 index was type specific in all sera tested.

Specificities by the 2/1 index of the MNT and the IHAT were approximately the same in recurrent HVH-1 and HVH-2 infections and primary HVH-2 infections. Thus, the IHAT, being easier to perform, more economical, and less time consuming, would appear to be the most useful test in serum assays of large populations for the presence of previous infections with HVH-2. An intermediate range of 2/1 indexes should be delineated in the IHAT, and then sera within this range could be typed by the indirect hemagglutinating inhibition test (2, 3) after cross-absorption with heterologous virus.

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